

Biopositive Effects of Low-Dose UVB on Epidermis: Coordinate Upregulation of Antimicrobial Peptides and Permeability Barrier Reinforcement

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Whereas high-dose ultraviolet B (UVB) is detrimental to the epidermal permeability barrier, suberythral doses of UVB are used to treat atopic dermatitis (AD), which is characterized by defective permeability barrier and antimicrobial function. As epidermal permeability barrier and antimicrobial peptide (AMP) expression are coregulated and interdependent functions, we hypothesized that suberythral doses of UVB exposure could regulate AMP expression in parallel with permeability barrier function. Hairless mice were exposed to 40 mJ cm⁻² UVB (about 1/2 minimal erythema dose) daily for 1 or 3 days. Twenty-four hours after the last exposure, epidermal barrier function was assessed and skin specimens were taken for western blotting, immunohistochemistry, and quantitative reverse transcription-PCR for mouse β -defensin (mBD)-2, mBD3 and cathelin-related antimicrobial peptide (CRAMP). mRNA levels of the vitamin D receptor (VDR), 1 α -hydroxylase and key epidermal lipid synthetic enzymes were also quantified. After 3 days of UVB exposure, acceleration of barrier recovery and augmentation in expression of epidermal differentiation markers (for example, involucrin and filaggrin) occurred in parallel with increased mBD2, mBD3, and CRAMP expression at both the mRNA and protein level. VDR, 1 α -hydroxylase, and the major epidermal lipid synthetic enzymes were also upregulated. When an inhibitor of 1 α , 25 dihydroxyvitamin D₃ formation, ketoconazole, was applied immediately after UVB exposure, the cutaneous vitamin D system was inhibited, which in turn blocked epidermal lipid synthesis, AMP expression, and permeability barrier homeostasis, suggesting that the beneficial effect of low-dose UVB depends, at least in part, on activation of the cutaneous vitamin D system. Our results provide new insights into the mechanisms whereby low-dose UVB comprises effective therapy for AD.

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INTRODUCTION

Atopic dermatitis (AD) is characterized by abnormal immune responses, impaired barrier function, and an increased susceptibility to bacterial and viral infections. Expression levels of both human β -defensin (hBD)-2 and LL-37 are significantly decreased in lesional skin (Ong *et al.*, 2002), that is, upregulation does not occur as in other inflammatory or

infectious dermatoses (de Jongh *et al.*, 2005). Accordingly, the increased susceptibility to colonization and invasion by microbial pathogens that occurs in AD patients could be related to a combined deficit in permeability barrier function, coupled with low antimicrobial peptide (AMP) expression. Accordingly, stratum corneum expresses a number of AMPs, such as β -defensins, cathelicidins, RNase 7, and psoriasin, which both recognize and kill invading microorganisms, while functioning as distal sensors of the innate immune system (Elias, 2007). Two important AMP families are the β -defensins (hBD1, 2, 3, and 4) and the cathelicidin carboxy-terminal fragment (LL-37), whose murine homologs include mBD3 (hBD2) and cathelin-related antimicrobial peptide (CRAMP) (LL-37), respectively. Two of these AMPs, hBD2 and LL-37, are expressed at low levels in normal skin, but higher levels are induced in wounded and/or diseased skin (de Jongh *et al.*, 2005), with the important exception of AD (Ong *et al.*, 2002). Both hBD2 and LL-37 are copackaged along with lipids within lamellar bodies, and apparently delivered to the stratum corneum interstices by lamellar body secretion (Oren *et al.*, 2003; Braff *et al.*, 2005). Acute permeability barrier perturbations also upregulate mBD3 and

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Abbreviations: AD, atopic dermatitis; AMP, antimicrobial peptide; CRAMP, cathelin-related antimicrobial peptide; FAS, fatty acid synthase; hBD, human β -defensin; HMG-CoA, HMG-CoA reductase; mBD, mouse β -defensin; MED, minimal erythema dose; SPT, serine-palmitoyl transferase; TEWL, transepidermal water loss; UVB, ultraviolet B; VDR, vitamin D receptor

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CRAMP expression (Aberg *et al.*, 2008). Such barrier perturbations initiate a cytokine cascade that upregulates not only permeability barrier repair, but also hBD2 generation (Liu *et al.*, 2002). In contrast, LL-37 expression is not regulated by cytokines, but rather by ligands of the vitamin D₃ receptor (Weber *et al.*, 2005; Elias, 2007), which also are known stimulators of epidermal differentiation (Bikle, 2004). Notably, certain AMP (that is, LL-37) is essential for the homeostasis of the permeability barrier (Aberg *et al.*, 2008). Together, these results suggest a close interrelationship between the permeability and antimicrobial barriers that could be regulated in part through inflammatory cytokine signaling (Elias and Choi, 2005; Elias, 2007).

Although narrowband UVB or UVA phototherapy is a mainstay of treatment, both natural and artificial ultraviolet B (UVB) irradiation are commonly employed in the treatment of AD (Jekler and Larko, 1988; Wulf and Bech-Thomsen, 1998). Although the benefits of UVB are attributed generally to its immunomodulating activity, we hypothesized here that suberythral UVB therapy could improve permeability barrier function and antimicrobial defense in parallel. In contrast, single, high doses of UVB (for example, ≥ 4 minimal erythema dose (MED)) instead are known to exert detrimental effects on permeability barrier function (Haratake *et al.*, 1997; Holleran *et al.*, 1997; Takagi *et al.*, 2004). Moreover, low-dose UV phototherapy is useful for the treatment of various skin disorders, including psoriasis and AD (Jekler and Larko, 1988; Wulf and Bech-Thomsen, 1998; Leenutaphong *et al.*, 2000). Our hypothesis is also supported by the observations that (1) 1.5 MED UVB-irradiated skin becomes relatively resistant to irritants (Lehmann *et al.*, 1991) and (2) irradiation with suberythral doses of UVB stimulates epidermal ceramide production (Wefers *et al.*, 1991; Farrell *et al.*, 1998). Recently, repeated low-dose UVB has also been demonstrated to provide a limited protective effect against subsequent UV-induced inflammatory responses (Narbutt *et al.*, 2007). As AMP expression is regulated by UVB irradiation (Seo *et al.*, 2001; Mallbris *et al.*, 2005; Gambichler *et al.*, 2006), we also hypothesized that expression of hBD2 and LL-37, the two barrier-linked AMPs that are decreased in AD, could be upregulated by suberythral UVB, further accounting for its therapeutic benefits.

UVB also promotes production of biologically active 1 α ,25-dihydroxy-vitamin D₃ [1,25(OH)₂D₃], which has various roles including induction of differentiation and regulation of apoptosis, in keratinocytes as well as immunomodulatory effects in the skin (Lehmann, 2005). The benefits of vitamin D₃ could be related to both its known effects on epidermal differentiation and lipid synthesis (Bikle *et al.*, 2004a, b), coupled with enhanced innate immunity (that is, increased toll-like receptor and AMP expression) (Mallbris *et al.*, 2005; Weber *et al.*, 2005; Schaubert *et al.*, 2007). We further hypothesized that vitamin D₃ could provide a link between the permeability barrier and antimicrobial barriers, hypothesizing that low-dose UVB irradiation could improve permeability barrier function in parallel with increased AMP expression, mediated through increased cutaneous vitamin D₃ production. To date, there are no published studies that

have assessed the effects of suberythral UVB on either permeability barrier homeostasis or epidermal AMP expression. Herein, we report that UVB irradiation upregulates permeability barrier function and AMP expression in parallel in hairless mouse epidermis, and that cutaneous vitamin D₃ plays a major role in mediating this process.

RESULTS

Low-dose UVB irradiation for 3 days improves barrier recovery

We first delineated the effects of suberythral (low-dose) UVB exposure on epidermal permeability barrier homeostasis. Hairless mice ($n=6$ in each group) were exposed to 40 mJ cm⁻² UVB (about 1/2 MED) once daily for either 1 or 3 days. Control mice were sham-irradiated with fluorescent light for same exposure time. There were no significant differences in basal transepidermal water loss (TEWL) among the 3-day or 1-day UVB and control groups (Figure 1a). However, after previous UVB irradiation for 3 days, tape-stripped skin showed significantly accelerated barrier recovery in comparison with control mouse skin 3 hours after tape-stripping ($P=0.011$; Figure 1b). In contrast, there were no significant differences in barrier recovery rates following a single UVB dose versus sham light-exposed mice ($P=0.090$, $P=0.052$, respectively; Figure 1b). Thus, repeated, short-term exposure to low-dose UVB significantly accelerates the kinetics of barrier recovery after acute insults.

Low-dose UVB irradiation augments AMP expression at protein and mRNA level

As the expressions of mBD3 and CRAMP change in parallel with permeability barrier status (Aberg *et al.*, 2008), we next assessed whether the expression of mBD2, mBD3, and CRAMP protein increase in murine epidermis after low-dose UVB exposure. Epidermis from 3-day UVB-exposed mice demonstrated a marked increase in immunostaining for mBD2, mBD3, and CRAMP than either control or 1-day UVB-exposed animals. Moreover, the UVB-induced increase in AMP expression was largely localized to the outer epidermis (Figure 2). These immunohistochemical data were

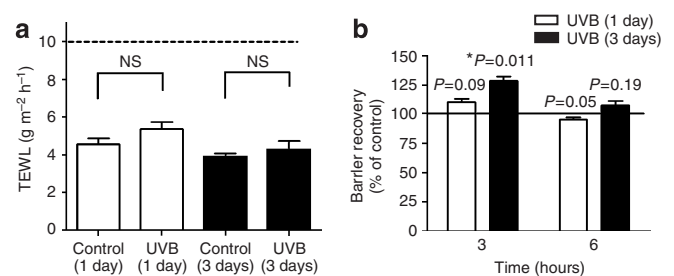


Figure 1. Low-dose UVB irradiation for 3 days increases barrier recovery rate after acute permeability barrier disruption by tape-stripping compared with sham light, without affecting basal TEWL. Hairless mice were exposed to 40 mJ cm⁻² UVB once daily for 1 or 3 days. Sham light was exposed on control group. (a) Basal TEWL rates and (b) barrier recovery rates following tape-stripping were measured. Statistical analysis was performed using an unpaired Student's *t*-test. Results are expressed as mean \pm SEM; $n=6$ in each group. (dotted horizontal line: upper limit of normal range; solid horizontal line: control level).

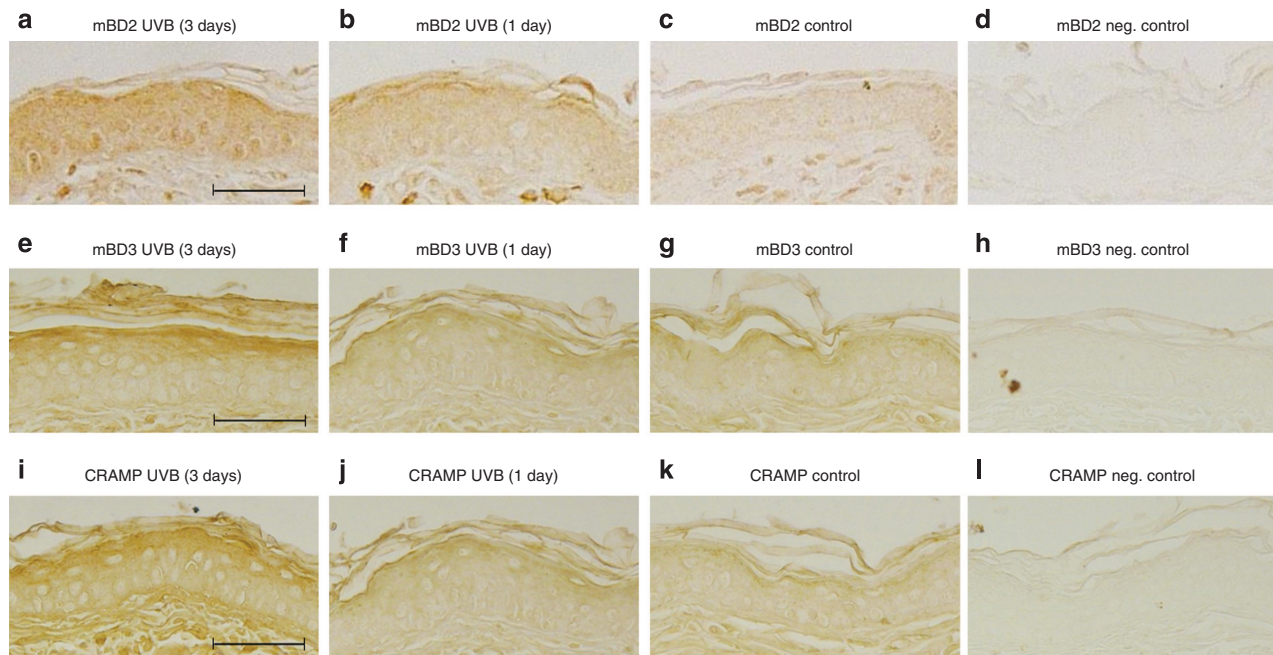


Figure 2. Low-dose UVB exposure increases expression of antimicrobial peptides in murine epidermis. Hairless mice ($n=6$ in each group) were exposed to 40 mJ cm^{-2} UVB once daily for either (a, e, i) 3 days or (b, f, j) 1 day. (c, g, k) Sham light was exposed on control group. (d, h, l) Negative controls were the skin samples stained without primary antibodies for mBD2, mBD3 and CRAMP. At 24 hours after the last irradiation, skin biopsies were taken and prepared for paraffin blocks. Immunohistochemical stains with diaminobenzidine (DAB) were used for (a-d) mBD2, (e-h) mBD3 and (i-l) CRAMP. Bar = $40 \mu\text{m}$.

confirmed by western blot analysis, which showed that levels of mBD2, mBD3, and CRAMP expression increased after 3 days of UVB exposure in comparison with either control or 1-day exposure (Figure S1). These results show that repeated suberythemal doses of UVB stimulate expression of AMP homologous of hBD2 and LL-37 in the outer epidermis of murine skin.

We next examined whether the changes in mBD2, mBD3, and CRAMP protein reflect increased expression at the transcriptional level, by assessing changes in epidermal RNA expression. Total RNA was extracted from epidermis and reverse-transcribed by standard methods, and expression was quantified by real-time reverse transcription-PCR (RT-PCR). Increased mRNA expression of mBD2, mBD3, and CRAMP was observed in 3-day UVB-irradiated mice compared with sham-light-irradiated controls (Figure S2a). Expression of mBD2 and CRAMP mRNA was more than twofold higher (216 and 248%, respectively) in 3-day UVB-irradiated epidermis than in controls, with the upregulation of mBD2 even more significant than for CRAMP (Figure S2b). In contrast, one-day UVB exposure did not upregulate either of these AMPs (Figure S2a). These results confirm that the UVB-induced increase in protein levels is reflected by a previous or concurrent increase in mRNA expression.

Low-dose UVB irradiation stimulates the cutaneous vitamin D₃ system

To ascertain whether 3-day exposure to low-dose UVB modulates expression of the cutaneous vitamin D₃ system, which is known to regulate LL-37 expression, epidermal

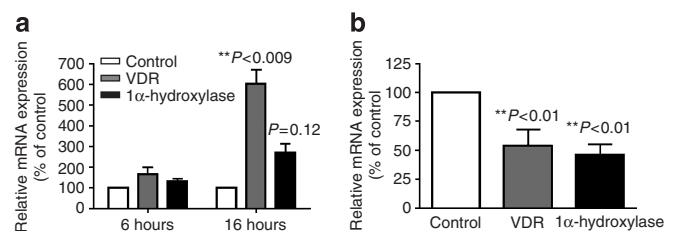


Figure 3. Low-dose UVB irradiation stimulates the cutaneous vitamin D₃ system, but this effect is inhibited by ketoconazole. Hairless mice ($n=3$ in each group) were exposed to 40 mJ cm^{-2} UVB once daily for 3 days. Sham light was exposed on control group. (a) At 6 or 16 hours after the last UVB exposure, expression of mRNA was quantified using a Rotor-Gene 3000 instrument system (Corbett Life Science) from the epidermis of dorsal skin. Relative mRNA expressions of vitamin D receptor (VDR) and 1 α -hydroxylase represent the activity of cutaneous vitamin D₃ system. (b) Immediately after each exposure, ketoconazole 2% cream or vehicle cream was applied to the irradiated dorsal skin. At 16 hours after the last exposure, expression of mRNA was quantified. Results are shown relative to the control mRNA levels.

mRNA levels of the vitamin D receptor (VDR) and 1 α -hydroxylase were quantified with real-time PCR. Sixteen hours after the last UVB exposure, there was a significant increase in mRNA for both of these genes, although the increase in 1 α -hydroxylase was less marked than for the VDR (mean levels of vitamin VDR and 1 α -hydroxylase mRNA increased by 604 and 270%, respectively, Figure 3a). Six hours after the last exposure, no significant differences in these genes were found in comparison with control groups (Figure 3a). Interestingly, mRNA levels of the vitamin D₃ system were much more increased after 16 hours than after

6 hours. These results indicated that low-dose UVB exposure results in upregulation of the cutaneous vitamin D₃ system.

Blockade of the cutaneous vitamin D system reverses the benefits of low-dose UVB on epidermal permeability barrier function and AMP expression

To elucidate whether the beneficial effects of low-dose UVB are mediated by UVB-induced activation of the cutaneous

vitamin D₃ system, we applied ketoconazole cream (2%) immediately after UVB exposure to block epidermal synthesis of active vitamin D₃ [(1,25(OH)₂D₃]. There was a significant decrease in mRNA for VDR and 1 α -hydroxylase (54 and 46%, respectively) in the ketoconazole- versus vehicle-treated epidermis, which indicated suppression of intra-cutaneous vitamin D₃ activity (Figure 3b). The mean basal TEWL in ketoconazole-treated mice was 14.4 g m⁻² hour⁻¹ (significant increase over the hydrobase-treated group was 10.3 g m⁻² hour⁻¹) (Figure 4a). The kinetics of barrier recovery also was slightly delayed in the ketoconazole- versus vehicle-treated mice at 3 hours ($P=0.09$) and 6 hours ($P=0.09$) (Figure 4b). In control, epidermal immunostaining of mBD2, mBD3, and CRAMP was less intense in ketoconazole-treated than in vehicle-treated mice (Figure 5). Together, these results indicate that the benefits of suberythemal doses of UVB on the barrier and antimicrobial defense are mediated by the cutaneous vitamin D₃ system.

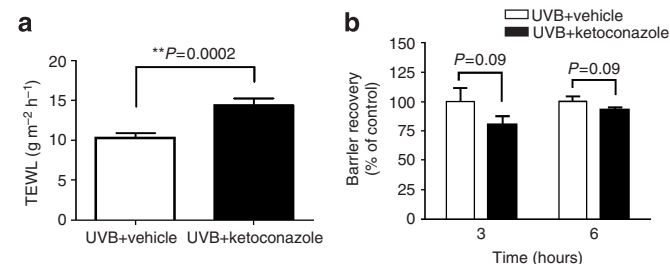


Figure 4. Inhibition of active vitamin D₃ synthesis by ketoconazole weakened the effect of low-dose UVB on the permeability barrier function. Hairless mice were exposed to 40 mJ cm⁻² UVB once daily for 3 days. Sham light was exposed on control group. Immediately after each UVB irradiation, 4 mg/cm² of 2% ketoconazole cream (Nizoral, Janssen) or vehicle cream (hydrobase) was topically applied to the irradiated dorsal skin. (a) Basal TEWL and (b) barrier recovery rates after acute barrier perturbation with tape stripping were measured at 16 hours after the last irradiation. Statistical analysis was performed using an unpaired Student's *t*-test. Results are expressed as mean \pm SEM; $n=5$ in each group.

Low-dose UVB irradiation increases both epidermal lipid synthetic enzymes and epidermal differentiation markers

We next assessed the basis for the UVB-induced improvement in barrier function. The changes in lipid synthetic enzymes, HMG-CoA reductase (HMG-CoA), fatty acid synthase (FAS), and serine-palmitoyl transferase (SPT), paralleled changes in the vitamin D₃ system (Figure 3a). The mRNA levels of the rate-limiting enzymes for epidermal lipid

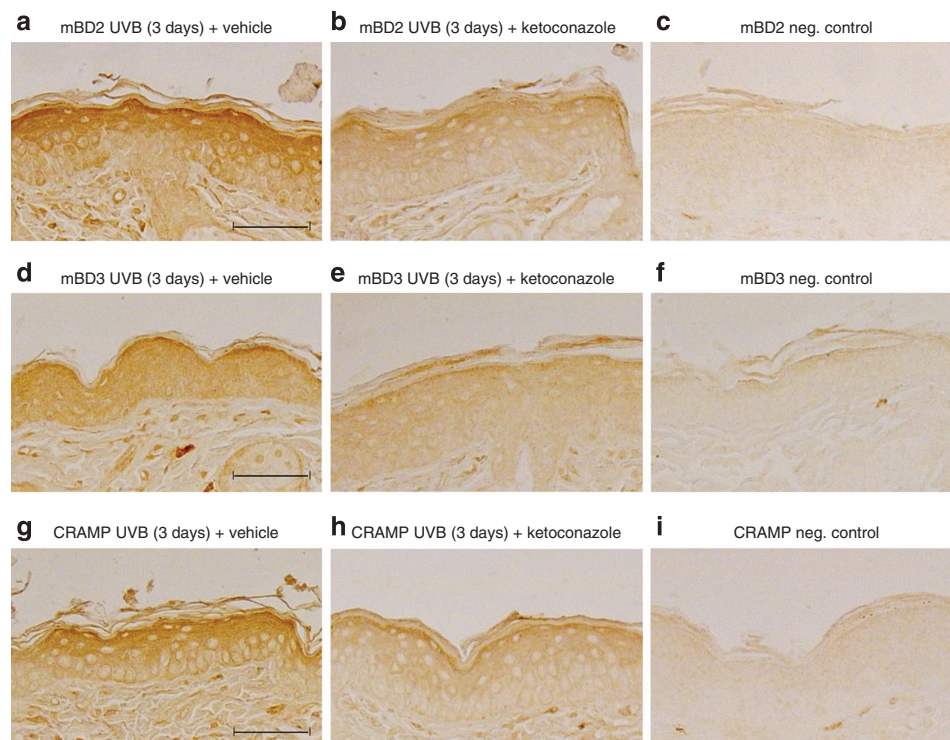


Figure 5. Inhibition of active vitamin D₃ synthesis by ketoconazole diminished the effect of low-dose UVB on the epidermal expression of antimicrobial peptides. Hairless mice ($n=3$ in each group) were exposed to 40 mJ cm⁻² UVB once daily for 3 days. Immediately after every exposure, (b, e, h) ketoconazole 2% cream or (a, d, g) vehicle cream was applied to irradiated dorsal skin. At 16 hours after the last irradiation, skin biopsies were taken and prepared for paraffin blocks. Immunohistochemical analysis was performed using DAB to stain (a-c) mBD2, (d-f) mBD3, and (g-i) CRAMP. (c, f, i) Skin immunostained for mBD2, mBD3, and CRAMP without primary antibody. Bar = 40 μ m.

synthesis such as FAS, SPT, and HMG-CoA increased by 340, 420, and 341%, respectively, at 16 hours (Figure 6a). Yet, at 6 hours, there was no significant increase in any of these enzymes. mRNA levels of the rate-limiting enzymes for the epidermal lipid synthetic enzymes, including FAS, SPT, and HMG-CoA, also significantly decreased in ketoconazole-treated epidermis (by 49.7, 70.4, and 52.2%, respectively, Figure 6b).

We next determined whether low-dose UVB induces the changes in the expression of two epidermal differentiation-related proteins, involucrin and filaggrin. Immunohistochemical staining for both of these proteins increased in the epidermis of suberythral UVB-exposed mice without the concurrent development of epidermal hyperplasia (Figure 7). The 3-day irradiated group showed more promi-

nent upregulation of involucrin and filaggrin than did 1-day-irradiated mice. These results indicate that UVB regulates not only epidermal lipid synthesis, but also epidermal differentiation, which provide a further mechanism for improved epidermal permeability and antimicrobial barrier function.

DISCUSSION

Owing to the apparent importance of barrier dysfunction and impaired antimicrobial defense in the pathogenesis of AD, we assessed here whether barrier function and AMP expression are inducible by repeated low-dose UVB exposure *in vivo*. Our results from real-time RT-PCR, western blot and immunohistochemical staining show that the expression of mBD2, mBD3, and CRAMP are markedly upregulated by 1/2 MED (40 mJ cm⁻²) of UVB irradiation daily over 3 consecutive days. Previous studies reported that a single dose of UVB upregulates expression of both hBD1 and hBD2 in human keratinocytes (Seo *et al.*, 2001), and a single MED dose of UVB upregulates hCAP18 mRNA in human skin (Mallbris *et al.*, 2005). In contrast, a 6-week course of narrowband UVB phototherapy with dose increment led to a decrease in cutaneous levels of hBD2 in patients with AD, whereas hBD3 did not change (Gambichler *et al.*, 2006). However, these results cannot be compared with ours, because Gambichler *et al.* evaluated AMP levels in full-thickness skin.

The molecular signaling mechanisms by which expression of AMP could be upregulated by UVB exposure remain poorly understood. β -Defensins are regulated by a variety of cytokines, whereas recent studies have shown that 1 α ,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] upregulates cathelicidin expression (Wang *et al.*, 2004; Mallbris *et al.*, 2005; Weber *et al.*, 2005; Schaubert *et al.*, 2007). It is well known that keratinocytes possess the capacity to synthesize a biologically

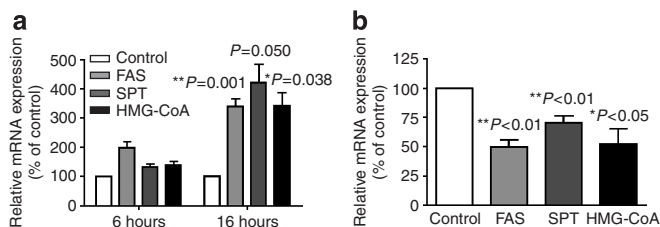


Figure 6. Low-dose UVB irradiation increases both epidermal lipid synthetic enzymes, but this effect is inhibited by ketoconazole also. (a) At 6 or 16 hours after the last UVB exposure, mRNA expression of rate-limiting enzymes of epidermal lipid synthesis including fatty acid synthase (FAS), serine-palmitoyl transferase (SPT), and HMG-CoA reductase (HMG-CoA) (b) was quantified using a Rotor-Gene 3000 instrument system (Corbett Life Science) from the epidermis of dorsal skin. (b) Immediately after each exposure, ketoconazole 2% cream or vehicle cream was applied to irradiated dorsal skin. At 16 hours after the last exposure, expression of mRNA was quantified. Results are shown relative to the control mRNA levels.

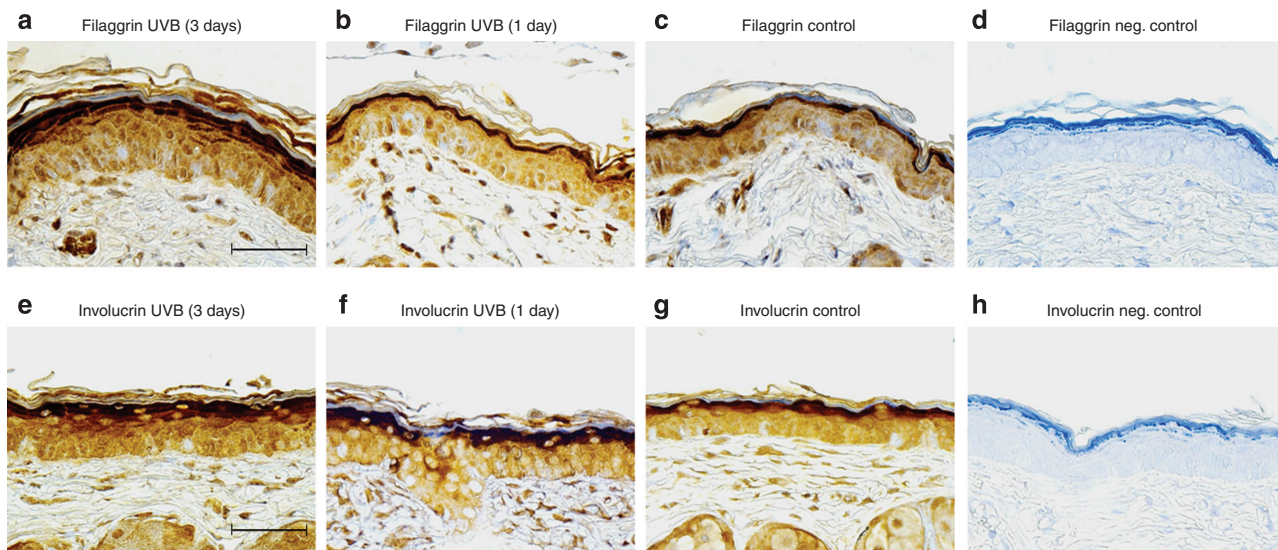


Figure 7. UVB exposure increases expression of differentiation markers in murine epidermis. (a-d) Filaggrin and (e-h) involucrin were immunostained using DAB ($n=3$ in each group). (a, e) Three-day UVB irradiated group showed more prominent expression compared with either (c, g) 3-day sham-light-irradiated control or (b, f) 1-day UVB-irradiated group. Negative controls were the skin samples stained without primary antibodies for (d) filaggrin and (h) involucrin. Bar = 40 μ m.

active form of vitamin D₃, 1,25(OH)₂D₃, through their own 1 α -hydroxylase (Bikle *et al.*, 2004a; Lehmann, 2005). But this pathway of 1,25(OH)₂D₃ production can be blocked by topical applications of ketoconazole, which inhibits cytochrome P450-dependent enzymes, such as the 1 α -hydroxylase (Lehmann *et al.*, 2003). Maximum cutaneous vitamin D₃ production after UVB exposure occurs at approximately 10–20% of the original epidermal 7-dehydrocholesterol concentration, a limit achieved with suberythemogenic UV exposures (Holick, 1981). As a result, a small amount of UVB exposure should suffice to generate synthesis sufficient vitamin D₃ to impact downstream events in epidermis.

Vitamin D action in peripheral tissues, such as the epidermis, is mediated by the VDR. Accordingly, the upregulation of epidermal VDR levels likely predicts enhanced effects of either exogenous/topical or intracutaneously generated 1,25(OH)₂D₃ in epidermis (Solvsten *et al.*, 1997; Mallbris *et al.*, 2005). Our study showed that mRNA levels for both the VDR and 1 α -hydroxylase are upregulated by 3-day UVB exposure with suberythemal dose, and conversely, down-regulated by blockade of vitamin D generation by ketoconazole. Hence, it is likely that considerable active 1,25(OH)₂D₃ is produced in response to suberythemal UVB, but ketoconazole likely lowered vitamin D₃ levels. Therefore, it is plausible that AMP upregulation induced by low-dose UVB is mediated through cutaneous production of 1,25(OH)₂D₃, the most active form of vitamin D₃.

We also showed that suberythemal doses of UVB irradiation improved permeability barrier homeostasis. In previous studies, permeability barrier was disrupted in murine epidermis with high doses of UVB irradiation (Haratake *et al.*, 1997; Holleran *et al.*, 1997), which was followed by a delayed increase in epidermal lipid synthesis as well as accelerated production and secretion of lamellar body, which eventually restore permeability barrier homeostasis (Holleran *et al.*, 1997). In our study, only 1/2 MED of UVB was employed daily. Although this dose provokes neither clinically evident inflammation nor barrier disruption, an increase in mRNA levels for HMG-CoA, FAS, and SPT as key synthetic enzymes of cholesterol, fatty acid, and sphingolipid resulted, which could account for the ability of low-dose UVB to improve permeability barrier homeostasis.

Downstream effects of increased endogenous vitamin D₃ production could also explain, in part, the benefits of low-dose UVB for barrier function. Previous studies have shown that 1,25(OH)₂D₃ likely plays an important role in the generation and maintenance of the permeability barrier and in epidermal differentiation (Bikle *et al.*, 2004a,b). And, 1,25(OH)₂D₃ increases expression of major epidermal differentiation proteins, such as involucrin, loricrin, filaggrin, and transglutaminase, as well as stimulates cornified envelope formation, which are required for optimal epidermal differentiation and, therefore, permeability barrier homeostasis (Bikle *et al.*, 2004a). Also, topical 1,25(OH)₂ vitamin D₃, calcitriol increases the lipid content of murine epidermis and counteracts the permeability barrier abnormality induced by topical steroids (Oh *et al.*, 2007). Our immunohistochemical assessment of murine epidermis after UVB irradiation for

3 days revealed an augmentation in involucrin and filaggrin expression, suggesting that suberythemal doses of UVB may stimulate not only lipid synthesis, but also corneocyte formation. Thus, low-dose UVB irradiation may result in an overall enhancement of barrier function through a variety of mechanisms.

Acute exposure to UV light induces a variety of cutaneous responses that can be either beneficial or harmful. This study clearly shows the beneficial effects of UVB, including increased epidermal AMP expression and permeability barrier reinforcement. Exposure dose is an important factor in determining the effects of UVB exposure. UV-induced DNA damage and barrier disruption increase linearly with increasing dose (Lamaud and Schalla, 1984). Indeed, previous studies have shown that a treatment regimen of repeated suberythemogenic doses is more effective for AD than conventional dose increments, in part because lower doses of UVB could reduce UV-induced adverse effects, such as erythema, xerosis, and inflammation, without increasing tanning and inflammation (Wulf and Bech-Thomsen, 1998). Our studies suggest that suberythemal doses of UVB not only have an acceptable side-effect profile, but they also induce beneficial effects on barrier function and antimicrobial defense, presumably mediated by vitamin D₃ production, leading to both epidermal lipid/DNA synthesis and AMP expression. However, further studies are needed to determine whether the local tissue concentrations of UVB-induced AMP are within an effective antimicrobial range, and whether UVB exposure enhances barrier function and antimicrobial defense in AD as well.

In conclusion, our results demonstrate beneficial effects of low-dose UV irradiation on the skin and provide indirect evidence for a possible connection between the epidermal permeability barrier and antimicrobial defense, which, at least in part, is mediated by cutaneous vitamin D₃ activation. The positive effects of low-dose UVB radiation on AMP expression and permeability barrier homeostasis can explain why UVB could be a useful therapeutic strategy for AD.

MATERIALS AND METHODS

Animals and UVB irradiation

Female hairless mice (8–12 weeks old) were purchased from the animal laboratory of Yonsei University. Mice were kept under controlled humidity (40%) and temperature (22 \pm 2 $^{\circ}$ C). All animal experiments described in this study were conducted in accordance with accepted standards of humane animal care, under protocols approved by the local animal research committee at Yonsei University Wonju College of Medicine. To observe the changes in AMP expression after UVB irradiation, the dorsal skin of each mouse was irradiated with 40 mJ cm⁻² UVB, equivalent to 1/2 MED, once a day for 1 or 3 days (n =6 each for functional study; n =3 each for immunohistochemistry, western blot, and PCR studies). UVB irradiation was delivered with Philips TL20W/12 fluorescent lamps (Eindhoven, Netherlands) emitting 280–320 nm. One MED, previously determined using the same species, equals approximately 80 mJ cm⁻². A control group was exposed to sham light (ordinary fluorescent lamp, DULUX-L, Osram Korea, Ansan, Korea) for the same exposure time. Epidermal barrier function was assessed and

skin specimens for analysis of epidermal AMP and differentiation marker expression were obtained from the central dorsum 24 hours after the last exposure. Additionally, to assess the effect on cutaneous vitamin D₃ synthesis and mRNA expression of epidermal lipid synthesis-related enzymes, dorsal skins of mouse were obtained at 6 or 16 hours after 3-day UVB exposure. Finally, to elucidate the effect of vitamin D activated by UVB on epidermal permeability and antimicrobial barrier, ketoconazole (known as an inhibitor of 1,25(OH)₂D₃ synthetic enzymes) was used. A quantity of 4 mg cm⁻² of a cream containing 2% ketoconazole (Nizoral[®], Janssen Pharmaceutica, Titusville, NJ) or vehicle cream (hydrobase) as control were topically applied to the skin immediately after each UVB irradiation for 3 days as reported previously (Lehmann *et al.*, 2003). Functional study for permeability barrier, immunohistochemical stain for AMP, and real-time RT-PCR for vitamin D system and epidermal lipid enzyme were done as described.

Assessment of epidermal permeability barrier function

To determine whether UVB irradiation influenced the kinetics of barrier recovery, we first assessed baseline permeability barrier status and recovery after acute disruption by tape-stripping. The dorsal surface of the skin was disrupted by tape-stripping with cellophane until TEWL levels reached 50 g m⁻² hour⁻¹ (normal basal TEWL <10 g m⁻² hour⁻¹), and measurements were repeated 3 and 6 hours following tape-stripping using a Tewameter TM210 (Courage and Khazaka, Cologne, Germany). The barrier recovery rate was expressed as percent recovery calculated by the following formula: (TEWL immediately after barrier disruption–TEWL at the indicated time point)/(TEWL immediately after barrier disruption–baseline TEWL) × 100%.

Immunohistochemical staining

For the light microscopic examination, full-thickness skin specimens were fixed in 10% formalin solution and embedded in paraffin. Sections of 5 μm thickness were cut and stained with antibodies for the mBD2 (SantaCruz Technology, Santa Cruz, CA), mBD3 (Santa Cruz), and CRAMP (Santa Cruz) as AMP, and filaggrin (Covance, Berkeley, CA) and involucrin (Covance, CA) as differentiation markers.

After deparaffinization, sections were rehydrated sequentially with 100, 90, and 70% ethanol and incubated for 5 minutes in Tris-buffered saline to inactivate endogenous peroxidases. Samples were then blocked for 10 minutes with blocking serum solution (DAKO, Carpinteria, CA) and incubated overnight at 4°C with primary antibody. After several washes in Tris-buffered saline, they were incubated for 30 minutes with a secondary biotinylated antibody. Antigen was detected with the avidin-biotin complex system (Vector, Burlingame, CA), according to the manufacturer's instructions by using 3,3'-diaminobenzidine tetrahydrochloride as substrate. Then they were examined under a light microscope.

Total RNA isolation and cDNA synthesis

Skin was excised from the irradiated area. Subcutaneous fat was removed with a surgical blade and epidermis was obtained by EDTA (Sigma, St Louis, CA) separation. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA), resuspended in RNase-free water, and quantified by means of UV spectrophotometer (Perkin Elmer Ltd, Shelton, CT). Single-stranded cDNA was prepared from

1 μg of total RNA in a 20 μl reaction volume using Oligo-dT primer (TaKaRa, Osaka, Japan), which contains 5 mM MgCl₂, 1 mM dNTP mixture, 1 U μl⁻¹ RNase inhibitor, and 0.25 U μl⁻¹ AMV reverse transcriptase.

Classic RT-PCR

PCR was performed using primers for AMP (Table S1). Primers were designed based on retrieved GeneBank sequences using Primer 3 software (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi/>, Massachusetts Institute of Technology, Cambridge, MA). Each reaction was prepared with a *Taq* DNA polymerase buffer, 0.2 mM of each deoxy-NTP, 2.5 mM MgCl₂, 0.2 mM of each forward and reverse primer, 5 U μl⁻¹ *Taq* DNA polymerase, and 2 μl cDNA, in a total volume of 20 μl. Reactions were cycled at 94°C for 5 minutes, followed by 35 cycles of 1 minute at 94°C, 1 minute at the annealing temperature (Table S1), and for 2 minutes at 72°C. All RT-PCR products were subsequently separated by electrophoresis on 2% agarose gels containing 0.5 μg ml⁻¹ ethidium bromide and visualized with UV light.

Quantitative real-time PCR

Real-time RT-PCR was performed using the LightCycler instrument (Roche Diagnostics, Mannheim, Germany) for mBD2 and CRAMP measurement and Rotor-Gene 3000 (Corbett Life Science, Sydney, Australia) for VDR, 1α-hydroxylase, FAS, SPT, and HMG-CoA, according to the manufacturer's instructions.

Briefly, real-time PCR was carried out by rapid cycling in a reaction volume of 20 μl. The reaction mixture consisted of a master mix containing *Taq* DNA polymerase, dNTP mixture, and reaction buffer (LightCycler-FastStart DNA Master Hybridization Kit) (Roche Diagnostics, Mannheim, Germany). In all the assays, 5 mM MgCl₂, 0.5 mM of each primer (Table S2), 0.2 mM of each probe, and 2 μl of template cDNA were used. The amplification and detection were carried out as follows: after an initial preincubation and denaturation step of 10 minutes at 95°C, amplification was performed in a three-step cycle procedure (denaturation 95°C, 10 seconds; annealing 60°C, 10 seconds; and elongation 72°C, 5 seconds) for 55 cycles and final cooling. Quantification of target gene expression was obtained by direct comparison with external standards amplified in parallel reactions in the same run. The LightCycler instrument system needs two fluorescence resonance energy transfer probes, but the Rotor-Gene 3000 system use a dual-labeled probe. Sequence-specific probes were designed depending on each PCR machine system. The sequences of primers and hybridization probes are listed in Table S2.

Western blot analysis

For western blotting of mBD2, mBD3, and CRAMP, the peptides were extracted from epidermis separated from full-thickness mouse skin. The proteins were separated by 4–12% SDS-PAGE with 3[N-morpholino]propanesulfonic acid (MOPS) buffer (Invitrogen) and transferred to polyvinylidene difluoride membranes. After blocking with 5% non-fat dry milk, membranes were incubated overnight at 4°C with anti-mBD2, anti-mBD3, or anti-CRAMP antibodies (SantaCruz Technology, Santa Cruz, CA) diluted 1:200. After several washes, peroxidase-conjugated anti-goat antibody was added (final dilution 1:2,000) for 1 hour at room temperature. Signals was detected using the ECL Plus Western blotting Detection System (Amersham, Buckinghamshire, UK).

Statistical analysis

Data are presented as mean \pm SEM. The unpaired Student's *t*-test was used for analysis of differences between two groups. **P* < 0.05 was considered statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPLEMENTARY MATERIAL

Table S1. Classic RT-PCR primers of antimicrobial peptides.

Table S2. Oligonucleotide primer and hybridization probe sequences for real-time PCR.

Figure S1. Western immunoblotting reveals increased bands of mBD2, mBD3, and CRAMP in 3-day UVB-irradiated murine epidermis.

Figure S2. mRNA expression of mBD2, mBD3, and CRAMP are increased after 3-day UVB exposure.

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